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Ochratoxin A – Determination of ochratoxin A in serum by CE-LIF

Biomonitoring Method – Translation of the German version from 2020

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Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed the presented biomonitoring method.

The method enables the determination of ochratoxin A in small volumes of serum. The samples are purified by liquid-liquid extraction and the analyte is extracted from the acidified sample with dichloromethane. After the extraction agent has been removed, the residue is dissolved in water. The extracts are injected into the capillary, then the individual components are separated by capillary electrophoresis and detected by laser-induced fluorescence. Coumarin-3-carboxylic acid is used as an internal standard.

Calibration standards are prepared in serum and processed in the same way as the samples to be analysed.

1 Characteristics of the method

Matrix	Serum
Analytical principle	Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF)

Parameter and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
Ochratoxin A	303-47-9	Ochratoxin A	303-47-9

Reliability data

Ochratoxin A

Within-day precision:	Standard deviation (rel.)	s_w = 20.5%, 11.6%, or 4.90%
	Prognostic range	u = 88.2%, 49.9%, or 21.1%
	at a spiked concentration of 0.5 µg, 2.0 µg, or 10.0 µg ochratoxin A per litre of serum and n = 3 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 27.9%, 17.0%, or 6.90%
	Prognostic range	u = 120%, 73.2%, or 29.7%
	at a spiked concentration of 0.5 µg, 2.0 µg, or 10.0 µg ochratoxin A per litre of serum and n = 3 determinations	
Accuracy:	Recovery rate (rel.)	r = 95%
	at a spiked concentration of 5.0 µg ochratoxin A per litre of serum and n = 4 determinations	
Detection limit:	0.2 µg ochratoxin A per litre of serum	
Quantitation limit:	0.6 µg ochratoxin A per litre of serum	

2 General information on ochratoxin A

Ochratoxin A is a naturally occurring mycotoxin, which was first isolated and chemically characterised in 1965 (Figure 1).

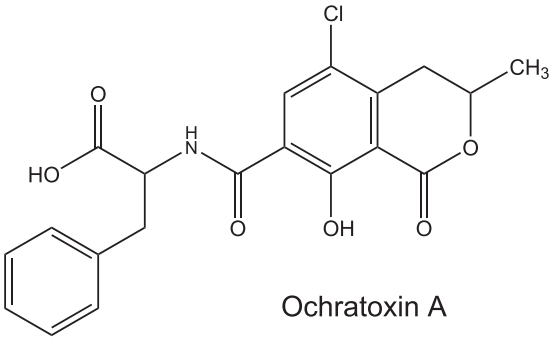

Fig. 1 Structure of ochratoxin A

Ochratoxin A is produced by different fungal species of the Penicillium and Aspergillus genera (*Aspergillus ochraceus, Aspergillus carbonarius, Penicillium verrucosum*), which are common and widespread in nature. Mycotoxins such as ochratoxin A are produced during plant growth, but in most cases, they are produced later on during the storage of plant material. Accordingly, ochratoxin A can be found in feed for farm animals as well as in food intended for human consumption.

As far as human nutrition is concerned, ochratoxin A is mainly found in cereals and cereal products (Rizzo et al. 2002), dried fruits, spices (Scheuer and Gareis 2002), nuts (Overy et al. 2003), coffee (Joosten et al. 2001; Taniwaki et al. 2003), and wine (Battilani and Pietri 2002; Visconti et al. 1999). However, due to its presence in animal feed, ochratoxin A levels in pork, poultry, and cheese may also contribute to human exposure (CONTAM 2019).

In the European Union, Commission Regulation (EC) No. 1881/2006 (European Commission 2006), sets maximum levels for ochratoxin A in plant-based foods between 0.5 µg/kg and 20 µg/kg – with the exception of liquorice extract. The European Food Safety Authority (EFSA) does not currently set a tolerable daily intake (TDI) for ochratoxin A due to the carcinogenic and genotoxic effects of the substance (CONTAM 2019).

In humans, ingested ochratoxin A can be detected in blood serum (Malir et al. 2016; Thuvander et al. 2001) and in urine (Malir et al. 2016). The fact that ochratoxin A can be detected in almost all human blood sera, including those in Germany, suggests that consumers are consistently exposed to ochratoxin A by both plant- and animal-based foods (Märtlbauer et al. 2009).

While the non-occupationally exposed general population is exposed to ochratoxin A mainly through diet, at the workplace inhalation of ochratoxin A-containing dusts is also possible. Studies on workers have shown that increased ochratoxin A exposure can occur in food production (Degen et al. 2007), food processing (Iavicoli et al. 2002), and waste management (Degen et al. 2003) (Table 1).

Tab. 1 Ochratoxin A in serum following occupational exposure

Occupational sector (country)	Number of persons n	Ochratoxin A in serum [µg/l]			References
		Mean	Median	Range	
Malting/barley – July (Germany)	7	–	0.34	0.13–2.60	Gareis and Meussdoerffer 2000
Malting/barley – October (Germany)	7	–	0.58	0.49–0.93	
Controls/general population (Germany)	74	–	0.21	0.07–0.91	
Food processing (coffee, cocoa, spices) (Italy)	6	2.29 ± 0.99	–	0.94–3.28	Iavicoli et al. 2002
Controls (Italy)	23	0.33 ± 0.25	–	0.03–0.95	
Waste deposition (EU)	76	–	0.36	n. s.[a)]–1.65	Degen et al. 2003
Waste sorting and recycling (EU)	60	–	0.53	n. s.–2.89	
Controls (EU)	84	–	0.39	n. s.–2.00	
Grain farming (Norway)	106	0.37	–	0.021–2.84	Skaug 2003
Controls (Norway)	104	0.42	–	0.036–5.53	
Cargo handling (grain, green coffee) (Germany)	9	0.53 ± 0.30	0.42	0.14–1.04	Degen et al. 2005
Grain silos (barley, wheat, rice, oats) (Germany)	61	0.28 ± 0.13	0.26	0.07–0.75	Degen et al. 2007

[a)] not specified

After oral intake, ochratoxin A is rapidly absorbed and is almost completely bound to albumin or other serum proteins in the human body. This results in a long elimination half-life of up to 35 days. Ochratoxin A is mainly metabolised to ochratoxin-alpha and is excreted as glucuronide. In humans, ochratoxin A and its metabolites are primarily excreted via the kidneys, which are also the main target organs for ochratoxin A toxicity (CONTAM 2019).

The Commission has classified ochratoxin A in Carcinogen Category 2 and Category 3 B for germ cell mutagens (DFG 2020). The IARC (International Agency for Research on Cancer) has classified ochratoxin A as possibly carcinogenic to humans (group 2B) (IARC 1993). For further details on the toxicological evaluation of ochratoxin A, please refer to the relevant MAK documentation (Greim 2006) and the EFSA publication (CONTAM 2019).

The method presented herein has already been published internationally (Köller et al. 2004).

3 General principles

This method allows the determination of ochratoxin A in small volumes of serum. The samples are purified by liquid-liquid extraction and the analyte is extracted from the acidified sample with dichloromethane. The extract is evaporated to dryness and the residue dissolved in water. The extracts are introduced into the capillary, the individual components are separated by capillary electrophoresis and then detected by laser-induced fluorescence. Coumarin-3-carboxylic acid is used as an internal standard (ISTD).

Calibration standards are prepared in serum and processed in the same way as the samples to be analysed.

4 Equipment, chemicals and solutions

4.1 Equipment

- Capillary electrophoresis system (e.g. HP3D, Agilent Technologies Deutschland GmbH, Waldbronn, Germany)
- Helium-cadmium laser, 325 nm (e.g. Melles Griot 56 Series, IDEX Health & Science, New York, USA)
- Laser-induced fluorescence-detection system (LIF detector) (e.g. Picometrics ZetaLIF, ADELIS SAS, Grabels, France)
- Capillary column of fused silica with an inner diameter of 50 µm (e.g. Molex Polymicro™, Optronics GmbH, Kehl, Germany)
- Microcentrifuge (e.g. Galaxy 14D, VWR International GmbH, Darmstadt, Germany)
- Vortex mixer (e.g. Vortex 2, IKA-Werke GmbH & Co. KG, Staufen, Germany)
- pH meter (e.g. Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany)
- Precision balance (e.g. Sartorius AG, Göttingen, Germany)
- Beakers (e.g. Brand GmbH & Co. KG, Wertheim, Germany)
- 10-ml, 100-ml and 500-ml volumetric flasks (e.g. VWR International GmbH, Darmstadt, Germany)
- 10-ml volumetric pipette (e.g. Brand GmbH & Co. KG, Wertheim, Germany)
- Variably adjustable microlitre pipettes with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- 2-ml screw-cap glass vials incl. caps with PTFE septa (e.g. Agilent Technologies Deutschland GmbH, Waldbronn, Germany)
- 0.5-ml polypropylene reaction vessels (e.g. Eppendorf AG, Hamburg, Germany)
- 0.2-ml polypropylene vials (e.g. Agilent Technologies Deutschland GmbH, Waldbronn, Germany)
- Syringe filters, 0.45 µm (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)
- 4-ml serum monovettes (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be at least *pro analysi* grade.

- Acetonitrile (e.g. Merck KGaA, Darmstadt, Germany, No. 100017)
- Coumarin-3-carboxylic acid (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. C85603)
- Dichloromethane (e.g. Merck KGaA, Darmstadt, Germany, No. 100668)
- Disodium hydrogen phosphate heptahydrate (e.g. Merck KGaA, Darmstadt, Germany, No. 106575)
- Methanol (e.g. Merck KGaA, Darmstadt, Germany, No. 100837)
- Sodium chloride (e.g. Merck KGaA, Darmstadt, Germany, No. 106404)
- Sodium hydrogen carbonate (e.g. Merck KGaA, Darmstadt, Germany, No. 106329)

- Sodium hydroxide pellets (e.g. Merck KGaA, Darmstadt, Germany, No. 106469)
- Ochratoxin A from *A. ochraceus* (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. O3007)
- *ortho*-Phosphoric acid 85% (e.g. Merck KGaA, Darmstadt, Germany, No. 100552)
- *ortho*-Phosphoric acid 20% (e.g. VWR International GmbH, Darmstadt, Germany, No. KRAF20132.2000)
- Ultra-pure water (e.g. Milli-Q plus VE System (>18 MΩ), Merck KGaA, Darmstadt, Germany)
- Argon 5.0 (e.g. Linde GmbH, Pullach, Germany)

4.3 Solutions

All solutions are passed through a 0.45 µm syringe filter prior to use.

- Solution A

  Exactly 1.68 g of sodium hydrogen carbonate are weighed into a 100-ml volumetric flask and dissolved in ultra-pure water. The flask is then made up to the mark with ultra-pure water.

- Solution B

  Exactly 1.2 g of sodium chloride are weighed into a 10-ml volumetric flask and dissolved in about 2 ml of ultra-pure water. 1.3 ml of 85% *ortho*-phosphoric acid are then added and the flask is made up to the mark with ultra-pure water.

- Sodium hydroxide solution (1 mol/l)

  50 ml of ultra-pure water are placed in a 250-ml beaker. Exactly 4 g of sodium hydroxide pellets are added and dissolved by stirring. The solution is transferred to a 100-ml volumetric flask, rinsing the beaker with water. The flask is then made up to the mark with ultra-pure water.

- Sodium hydroxide solution (0.1 mol/l)

  10 ml of the 1 mol/l sodium hydroxide solution are placed in a 100-ml volumetric flask, which is then made up to the mark with ultra-pure water.

- Phosphate buffer (60 mmol/l, pH 7)

  Exactly 4.02 mg of disodium hydrogen phosphate heptahydrate are weighed into a 600-ml beaker and dissolved in 400 ml of ultra-pure water. The pH is adjusted to pH 7 by adding drops of 20% phosphoric acid. The solution is then transferred to a 500-ml volumetric flask, which is made up to the mark with ultra-pure water.

The solutions are stored at room temperature and are stable for at least three months under these conditions.

4.4 Internal standard (ISTD)

- ISTD stock solution (2900 mg/l)

  Exactly 29 mg of coumarin-3-carboxylic acid are weighed into a 10-ml volumetric flask and dissolved in acetonitrile. The flask is then made up to the mark with acetonitrile.

  The ISTD stock solution is stored at –20 °C and is stable for at least two months under these conditions.

- ISTD spiking solution (870 mg/l)

  330 µl of the ISTD stock solution are thoroughly mixed with 770 µl of ultra-pure water in a 2-ml glass vial.

The ISTD spiking solution is freshly prepared every workday and passed through a 0.45 µm syringe filter prior to use.

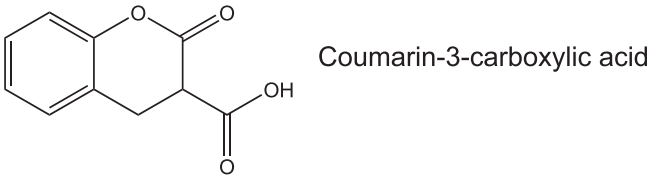

Fig. 2 Structure of the internal standard (coumarin-3-carboxylic acid)

4.5 Calibration standards

- Ochratoxin A stock solution (500 mg/l)

  5 mg of ochratoxin A are weighed exactly into a 10-ml volumetric flask and dissolved in methanol. The flask is then made up to the mark with methanol.

  The ochratoxin A stock solution is stored at –20 °C. Under these conditions, it is stable without loss of analyte for at least three months.

- Ochratoxin A working solution 1 (5 mg/l)

  100 µl of the ochratoxin A stock solution are pipetted into a 10-ml volumetric flask. The flask is then made up to the mark with ultra-pure water and the solution is thoroughly mixed.

- Ochratoxin A working solution 2 (250 µg/l)

  500 µl of ochratoxin A working solution 1 are pipetted into a 10-ml volumetric flask. The flask is then made up to the mark with ultra-pure water and the solution is thoroughly mixed.

The ochratoxin A working solutions can be used for a maximum of one week when stored in the refrigerator at 4 °C.

Spiking solutions in a concentration range between 0.5 µg/l and 250 µg/l are prepared by diluting the ochratoxin A working solution 2 with ultra-pure water. These spiking solutions are used to prepare the calibration standards by pipetting 25 µl of the spiking solution each to 100 µl of serum. The calibration standards prepared in this way are thoroughly mixed on the vortex mixer. Table 2 shows the pipetting scheme for the preparation of the calibration standards.

Tab. 2 Pipetting scheme for the preparation of calibration standards used to determine ochratoxin A in serum

Calibration standard	Ochratoxin A working solution 2 [µl]	Ultra-pure water [µl]	Concentration [µg/l]	
			Spiking solution	Calibration standard
1	10	990	2.5	0.5
2	20	980	5	1
3	50	950	12.5	2.5
4	100	900	25	5
5	200	800	50	10
6	500	500	125	25
7	1000	0	250	50

The calibration standards are freshly prepared every workday. Depending on the expected exposure levels, the concentration range of calibration can be adjusted. Usually, calibration up to 10 µg/l should be sufficient.

5 Specimen collection and sample preparation

5.1 Specimen collection

Blood samples are taken using serum monovettes. The monovettes are turned gently several times after blood collection and then allowed to stand at room temperature for at least 30 minutes. The blood cells are separated by centrifugation (10 minutes at 3000 rpm; 4 °C) and the serum is pipetted off.

5.2 Sample preparation

In a 0.2-ml polypropylene vial, 20 µl of solution A are pipetted to 20 µl of serum. The alkaline sample solution (pH > 9) is extracted twice with 100 µl dichloromethane each. The organic phase is pipetted off and discarded in each case. The solution is then acidified by adding 100 µl of solution B and extracted three times with 100 µl dichloromethane each. The extraction steps are performed using a vortex mixer for five minutes each. After extraction, the solution is centrifuged for 2.5 minutes at 14 000 × *g* to ensure better phase separation. After centrifugation, a three-phase system is formed, in which the upper aqueous phase is separated from the lower organic phase by the protein fraction appearing as a white ring. Each time, the organic phase is transferred into a vial with a conical insert. The combined extracts are evaporated to dryness under a stream of argon. The residue is dissolved in 20 µl of the ISTD spiking solution.

Unless a micro approach is required, larger sample volumes can be used.

6 Operational parameters

Analysis is performed using a capillary electrophoresis system coupled with a laser-induced fluorescence detector (CE-LIF).

The capillary electrophoresis system consists of a thermostated capillary, the ends of which are placed in a cathode and anode buffer reservoir. The electrodes connected to a high-voltage power supply are also immersed in these reservoirs. The detection window, which extends through the laser and photocell beam path, is located near the anode end. The signal from the detector is transmitted to a PC. Figure 3 shows the schematic setup of a CE-LIF system.

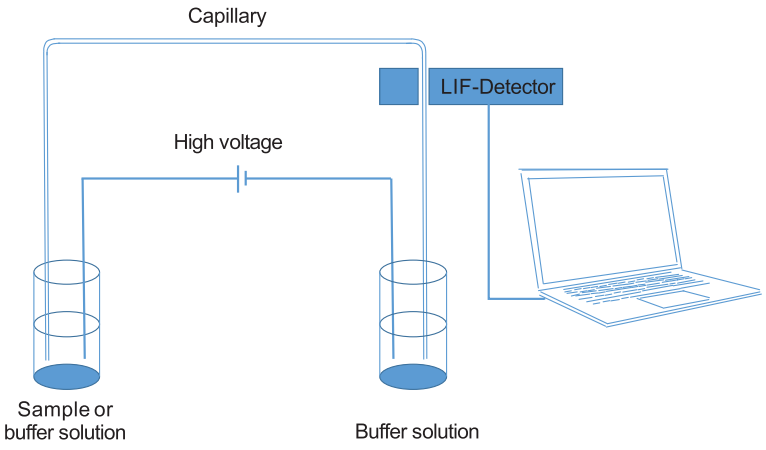

Fig. 3 Schematic setup of a CE-LIF system

6.1 Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF)

Capillary column:	Material:	Fused silica
	Length:	75 cm
	Effective length:	60 cm
	Inner diameter:	50 µm
	Temperature:	20 °C
LIF detector:	Helium-cadmium laser:	15 mW
	Excitation wavelength:	325 nm
Injection:	Hydrodynamic:	50 mbar for 30 s
	Stacking:	−20 kV for 30 s
	Separation:	+23 kV (constant)

All settings of the capillary electrophoresis system and of the detector are instrument-specific and must be adjusted individually by the user. The parameters given are therefore intended only as cursory guidance.

7 Analytical determination

After installation, the capillary is rinsed consecutively with methanol for 15 minutes, with ultra-pure water for 5 minutes, with 1 molar sodium hydroxide solution for 20 minutes, with 0.1 molar sodium hydroxide solution for 10 minutes, and finally with phosphate buffer for 20 minutes.

Furthermore, the capillary is rinsed for daily use with ultra-pure water for 10 minutes, with 1 molar sodium hydroxide solution for 10 minutes, with 0.1 molar sodium hydroxide solution for 10 minutes, and with phosphate buffer for 15 minutes. An aliquot of the samples, prepared as described in Section 5.2, is injected into the capillary. Injection is performed hydrodynamically for 30 seconds with a pressure of 50 mbar. Afterwards, a voltage of −20 kV is applied. After 30 seconds, the polarity of the electric current is changed by switching to +23 kV. The temporal variation is recorded for fluorescence excited at 325 nm. The respective electropherogram recorded by the detector shows a characteristic chart that depends on the sample composition, the type of injection, the detection, and further parameters.

Identification of ochratoxin A and of the ISTD is based on the migration times (Table 3). The analytical run time is about 15 minutes. After each measurement, the capillary is rinsed with 0.1 molar sodium hydroxide solution for one minute and with phosphate buffer for two minutes.

Tab. 3 Migration times for the determination of ochratoxin A in serum

Analyte	Migration time [min]
Ochratoxin A	~8.5
Coumarin-3-carboxylic acid	~9.6

The migration times given in Table 3 are intended only as cursory guidance. Users must ensure proper performance of the CE system used, which influences the resulting migration behaviour of the substances. Figures 4 and 5 show electropherograms of two sera spiked with 0.5 µg and 1 µg ochratoxin A per litre, respectively.

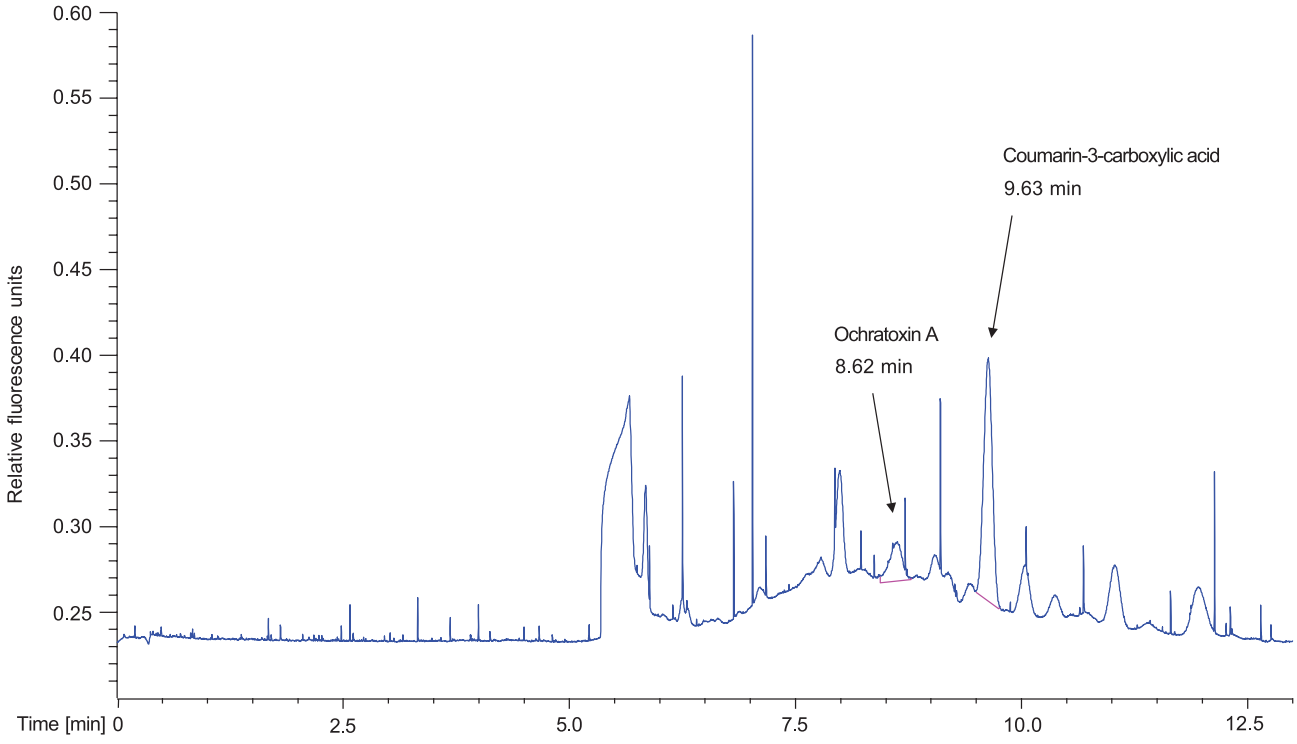

Fig. 4 Electropherogram of a serum sample spiked with 0.5 µg ochratoxin A per litre

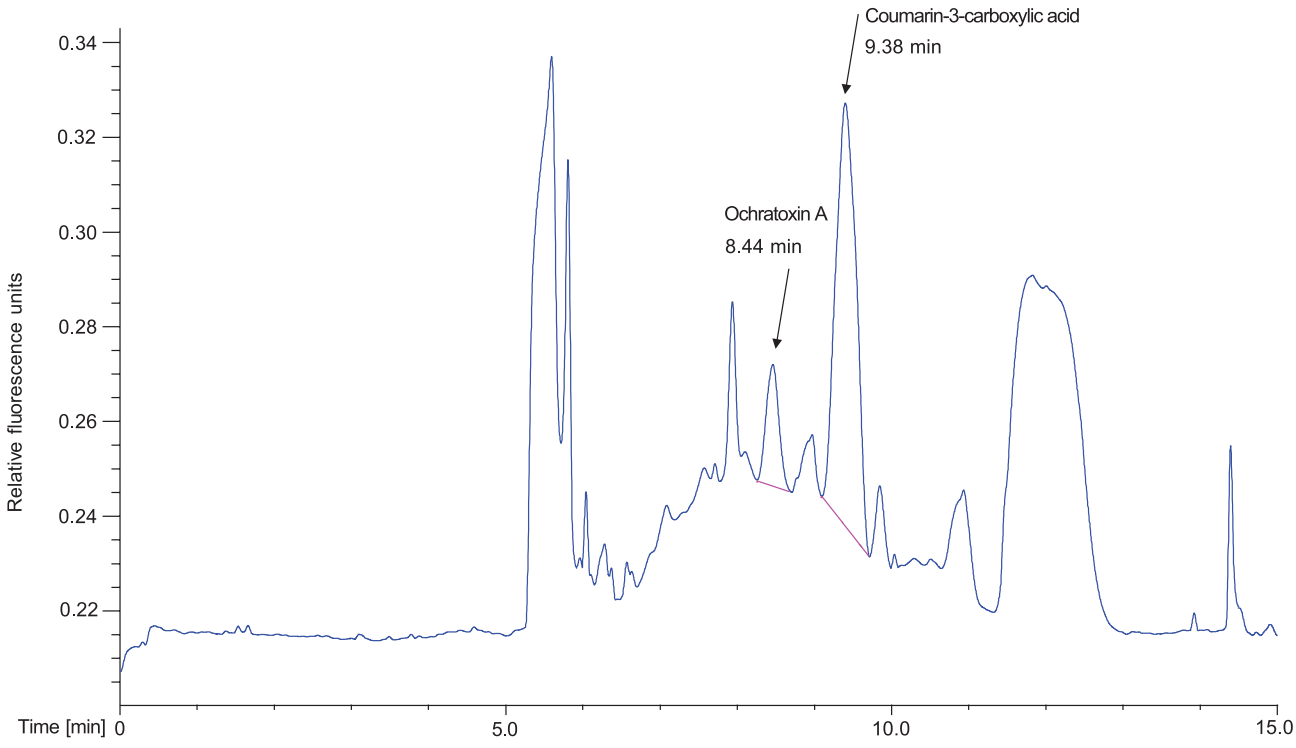

Fig. 5 Electropherogram of a serum sample spiked with 1 µg ochratoxin A per litre

8 Calibration

The calibration standards, prepared as described in Section 4.5, are processed in the same way as the samples to be analysed (cf. Section 5) and analysed by CE-LIF (cf. Section 7). Calibration graphs are obtained by plotting the quotients of the peak areas of the analyte and the internal standard against the concentration of the calibration standards. The calibration curve is linear under the described analytical conditions in the concentration range from 0.5 µg/l to 50 µg/l. As an example, Figure 6 shows a calibration curve for ochratoxin A in serum.

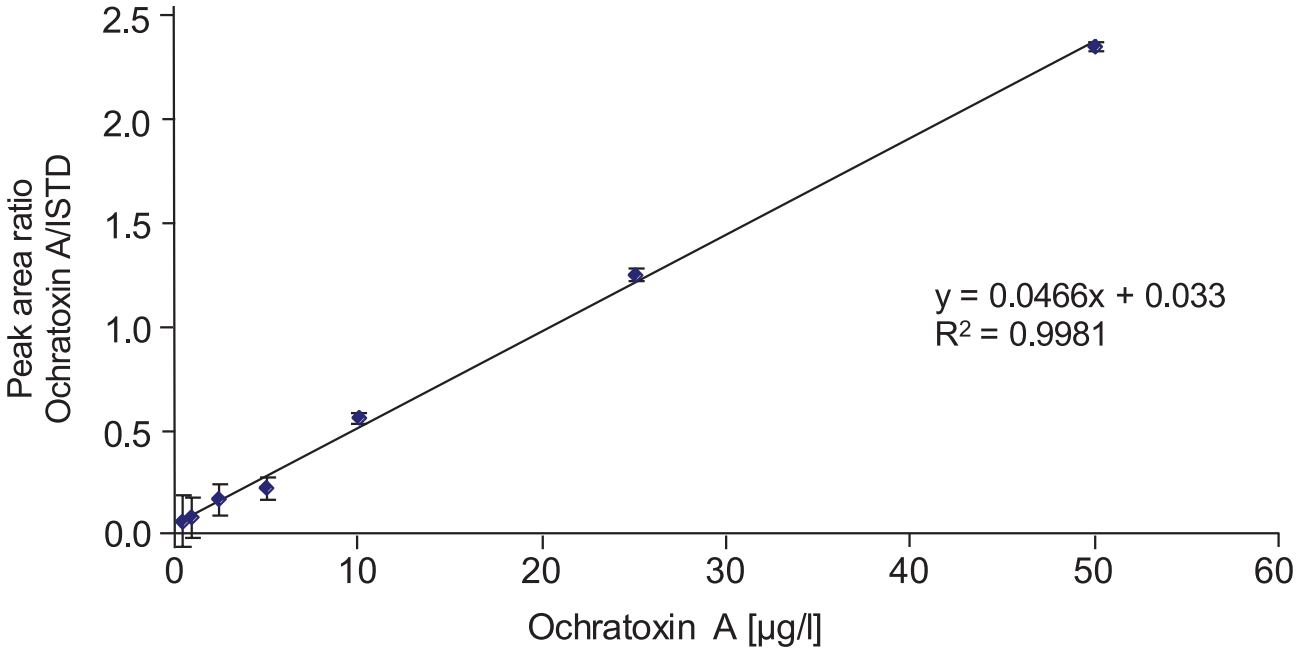

Fig. 6 Calibration curve for the determination of ochratoxin A in serum

Depending on the expected exposure levels, the concentration range of calibration can be adjusted. Usually, calibration up to 10 µg/l should be sufficient.

9 Calculation of the analytical results

The analyte concentrations in the serum samples are calculated using the calibration function pertaining to the corresponding analytical run (Section 8). The analyte concentration of a sample is determined by dividing the peak area of the analyte by the peak area of the internal standard. The quotient thus obtained is entered into the equation of the calibration curve established per Section 8 to give the respective analyte concentration in µg/l. Any reagent blank value has to be subtracted from the analytical results.

10 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter of the MAK Collection for Occupational Health and Safety (Bader et al. 2010; Bundesärztekammer 2014). To check precision, three quality-control samples with known analyte concentrations are analysed within each analytical run. As control material is not commercially available, it must be prepared in the laboratory. To this end, serum is spiked with the standard solution of the analyte at three different concentration levels, so that the concentration levels of the control materials are within the relevant concentration

range (e.g. 0.5 µg/l, 2.0 µg/l, 10 µg/l). Aliquots of these samples are stored at –20 °C and are included in each analytical run as quality-control samples. Care must be taken to ensure even distribution within the sequence. The nominal value and the tolerance ranges of the quality-control materials are determined in a pre-analytical period (Bader et al. 2010). A reagent blank is included in each analytical run in addition to the quality-control samples.

11 Evaluation of the method

The reliability of the method was verified by comprehensive validation and comparative measurements in a second, independent laboratory.

11.1 Precision

Within-day precision was determined using the quality-control samples spiked with 0.5 µg, 2.0 µg, and 10 µg ochratoxin A per litre of serum (see Section 10). The material was processed and analysed three times in parallel. The obtained within-day precision data are presented in Table 4.

Tab. 4 Within-day precision for the determination of ochratoxin A in serum (n = 3)

Analyte	Spiked concentration [µg/l]	Determined level [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Ochratoxin A	0.5	0.45	20.5	88.2
	2.0	2.1	11.6	49.9
	10.0	9.8	4.9	21.1

Day-to-day precision was determined by processing and analysing spiked serum samples on three consecutive days, using quality control samples spiked with 0.5 µg, 2.0 µg and 10 µg ochratoxin A per litre of serum (see Section 10). The obtained day-to-day precision data are presented in Table 5.

Tab. 5 Day-to-day precision for the determination of ochratoxin A in serum (n = 3)

Analyte	Spiked concentration [µg/l]	Determined level [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Ochratoxin A	0.5	0.45	27.9	120
	2.0	2.16	17.0	73.2
	10.0	10.5	6.9	29.7

11.2 Accuracy

Recovery was determined using an ochratoxin A solution with a concentration of 5.0 µg/l. The solution was processed and analysed four times, yielding recovery rates in the range from 86% to 103% (see Table 6).

Tab. 6 Mean relative recovery rate for the determination of ochratoxin A in serum (n = 4)

Analyte	Spiked concentration [µg/l]	Mean relative recovery [%]	Range [%]
Ochratoxin A	5.0	95	86–103

11.3 Limits of detection and quantitation

The detection limit and quantitation limit were determined on the basis of a signal-to-noise ratio of 3 : 1 for the detection limit and of 9 : 1 for the quantitation limit. The values calculated for the determination of ochratoxin A in serum are shown in Table 7.

Tab. 7 Limit of detection and limit of quantitation for the determination of ochratoxin A in serum (n = 6)

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
Ochratoxin A	0.2	0.6

11.4 Sources of error

With capillary electrophoresis, migration times may vary from one analytical run to another. In order to compensate for these drifts, which may occur due to the installation of a new capillary column or buffer consumption, the correction method published by Li et al. (2000) can be applied. Matrix components may also interfere with the migration times of the analyte or of the ISTD. In such cases, it is advisable to spike the sample with 5 µg ochratoxin A per litre and to repeat the analysis.

12 Discussion of the method

The described method allows the quantification of ochratoxin A in very small volumes of serum. Sample preparation by means of liquid-liquid extraction is simple and requires little time and effort. The analytical run time from one measurement to the next is about 15 minutes, so that three to four samples can be analysed per hour. The method is, however, not suitable for automation.

With capillary electrophoresis, the separation of the sample components is based on their different migration behaviour in a capillary, to which high voltage is applied. In the case of the applied capillary zone electrophoresis, a variant of CE, a homogeneous electric field is created by using a buffer solution. Detection is based on the induced fluorescence of the analytes passing through the detection window of the capillary. Due to the short light path, laser-induced fluorescence detection is clearly superior to other optical detection methods in terms of both specificity and sensitivity.

Despite the small sample volumes, a detection limit of 0.2 µg/l could be obtained by using a special injection technique (stacking). The precision data can be considered satisfactory. Unless a micro approach is required, larger sample volumes can be used. This should also facilitate sample preparation and processing, and further improve the precision of the method.

Due to the quantitation limit to be obtained, this method is only suitable for the detection of elevated ochratoxin A levels in environmental or occupational settings. The background exposure of the general population, which averages at 0.21 µg ochratoxin A per litre of serum in Germany (see Table 1) cannot be quantified by this method.

Instruments used HP3D capillary electrophoresis system with a Polymicro™ capillary column of fused silica (Molex Polymicro™, Optronics GmbH, Kehl, Germany); laser-induced fluorescence detection with helium-cadmium laser (Melles Griot 56 Series, 325 nm); and Picometrics ZetaLIF detector (Picometrics ZetaLIF, ADELIS SAS, Grabels, France).

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